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Rate and Lag of KNO_3-Induced Germination of Spores of the Food-Poisoning Anaerobe *Clostridium perfringens* Type A, NCTC 8238

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Temperature, pH, KNO_3 concentration, sodium phosphate buffer concentration and spore concentration affected the germination rate and germination lag of heat-activated spores of *Clostridium perfringens* type A, NCTC 8238. Optimal conditions for germination rate were pH 6.1 to 6.3, 32.5 to 35°C, 0.325 mg of spores per ml, 100 mM KNO_3, and 2 to 5 mM sodium phosphate. Both lag and rate of KNO_3-induced germination were the same under anaerobic or aerobic conditions.

The increasing recognition of the significance of the anaerobic sporeformer *Clostridium perfringens* type A and of its sporulation-associated enterotoxin (6) in food-poisoning outbreaks, particularly in institutional settings (4), has done much to stimulate research on the physiology of the spores of this organism.

Spores of certain food-poisoning strains of *C. perfringens* (1, 2), as well as of *Bacillus megaterium* QM B1551 (10, 14), are able to germinate in inorganic solutions, without organic adjuvants, K^+ being much more effective than Na^+ in supporting ionic germination (2, 10). In this paper, which constitutes part of our continuing program (11) to establish a data base on the activation, germination, and outgrowth of *C. perfringens* spores, comparable to that on *Bacillus*, we report on our investigations of optimal conditions for the KNO_3-induced germination of *C. perfringens* spores.

RESULTS AND DISCUSSION

In the following series of experiments, special attention was paid to a comparison of germination rate and germination lag under various conditions, these parameters of germination being determined as in Fig. 1.

pH. Germination rate increased from near undetectable at pH 5.4 to a peak of ca. 1.25 ± 0.2% optical density (OD) loss per min at pH 6.1 to 6.3, and then declined at higher pH (Fig. 2). Germination lag was ca. 13 min under standard conditions (pH 6.1), 27 min at pH 5.4, and 9.0 min at pH 6.5 to 7.5 (Fig. 2).

The extent of early germination, i.e., within the first 30 min (data not shown), was dependent on both rate and lag and had a pH optimum at ca. pH 6.2. However, spores continued germinating slowly and spore suspensions continued decreasing in OD, even under less than optimal conditions. Even spore suspensions with slow or delayed germination (at pH 5.7, for example) eventually attained an extent of germination equal to that achieved under the best conditions. This resulted in a broadening of the apparent optimal pH range, so that by 5 h, germination at pH levels from 5.7 to 6.3 had attained equivalent values, i.e., near 50% loss in OD. This pH range (5.7 to 6.3) is approximately that of meat and poultry, important vehicles (3–5) of *C. perfringens* food poisoning.

Temperature. Spores were incubated at various temperatures in the standard KNO_3 germination mixture (Fig. 1 legend) and, in some cases, were shifted after 1 h to a secondary incubation at 30°C (Table 1). The rate and extent (KNO_3-induced percent OD loss at 2 h) of germination appeared to be optimal at 32.5 to 35°C. Germination lag showed no clearly defined temperature optimum. Rather, with increasing temperature, there was a progressive decrease in the lag from >45 min at 15°C to <2 min at 45°C (Table 1). At the temperature where germination rate was maximal, the lag was ca. 8 to 10 min. The energy of activation (μ) for germination, calculated from an Arrhenius plot of reciprocal lags between 20 and 35°C was ca. 17.5 kcal (7.3×10^5 J), a value close to that for germination of *B. megaterium* spores in KNO_3 (10) or in glucose (13) and of a magnitude consistent with an enzymatic basis for KNO_3-induced germination of *C. perfringens* spores.

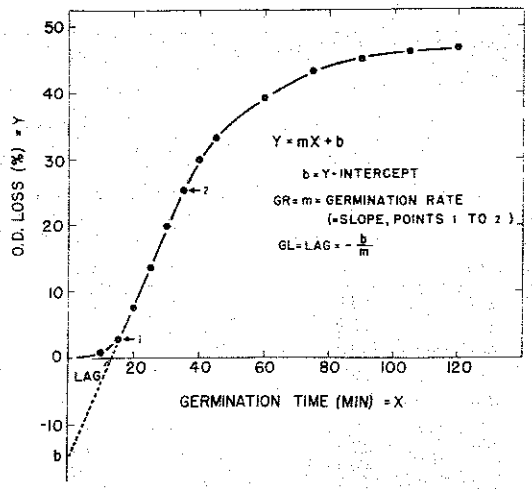

FIG. 1. *KNO_3-induced germination of heat-activated (65°C, 10 min) spores of* C. perfringens *NCTC 8238 under standard conditions.* C. perfringens *type A, NCTC 8238, used throughout these investigations, was isolated by Hobbs et al. (7) from salt beef and is classified as serotype 2 (8). Spore production, heat activation, glassware preparation, and chemicals were as previously described (11). Standard conditions for germination were 5 mM KNO_3, 10 mM sodium phosphate buffer, pH 6.1, 0.325 mg of spores per ml (OD, ca. 1.0; 1.1 × 10^8 spores per ml), and 30°C. No attempt was made to maintain anaerobiosis. Sodium phosphate was used because spores germinated readily in potassium phosphate buffer. OD (560 nm; disposable 13 by 100 mm Pyrex glass tubes; Bausch & Lomb Spectronic 21 colorimeter) was followed during incubation (11, 12). Each experiment examining a variable (pH, temperature, and spore, KNO_3, and buffer concentrations) was accompanied by two controls: one in the appropriate sodium phosphate buffer (without KNO_3) and the other under standard conditions. KNO_3-induced OD loss was corrected by subtracting OD loss in the buffer control. OD loss in buffer controls (10 mM sodium phosphate, pH 6.1 to 6.3; no KNO_3), at various temperatures, is shown in Table 1. The slope* (m) *of the least-squares regression line between points 1 and 2 (the rectilinear portion of the plot) represents germination rate (GR) and is expressed as percent OD lost per minute. The intercept of the regression line with the x-axis (no OD loss) represents germination lag (GL), which can be calculated from the regression line equation as* −b/m, *where* b *is the y-intercept of the regression line.*

The rate of OD loss (germination rate) was slightly higher at 40°C than at 23.5°C (Table 1). After 30 to 40 min of incubation, however, the rate at 40°C had begun to decrease, although the rate at 23.5 or 30°C was still maximal. As a result, the extent of germination at 2 h was substantially lower at 40°C than at 23.5°C. At 15°C, the rate of KNO_3-induced germination was low, but there was appreciable OD loss after 2 h, and *total* germination, as determined by phase-contrast microscopy, had reached about 20% (including those spores which had germinated in buffer without KNO_3). Even at 0°C, there were some 10% phase-dark spores after 3 h of incubation, suggesting that *C. perfringens* spores may germinate in foods even under refrigeration.

Spores, first incubated with KNO_3 at 0°C for 1 h, had lost virtually none of their ability to germinate at 30°C; their rate of OD loss after the shift to 30°C was only slightly less than that of spores kept continuously at 30°C (Table 1). Spores incubated with KNO_3 at 45 or 50°C had almost identical germination rates after shifting to 30°C (Table 1); this rate was lower than that of spores initially at 0°C or at 60°C for 1 h (Table 1). Why did spores, incubated in KNO_3 for 1 h at 60°C, germinate at 30°C at a higher rate than spores which were in KNO_3 for 1 h at 50°C before being shifted to 30°C? The following interpretation seems plausible. In 1 h at 50°C, 22% of the spores had germinated, i.e., became

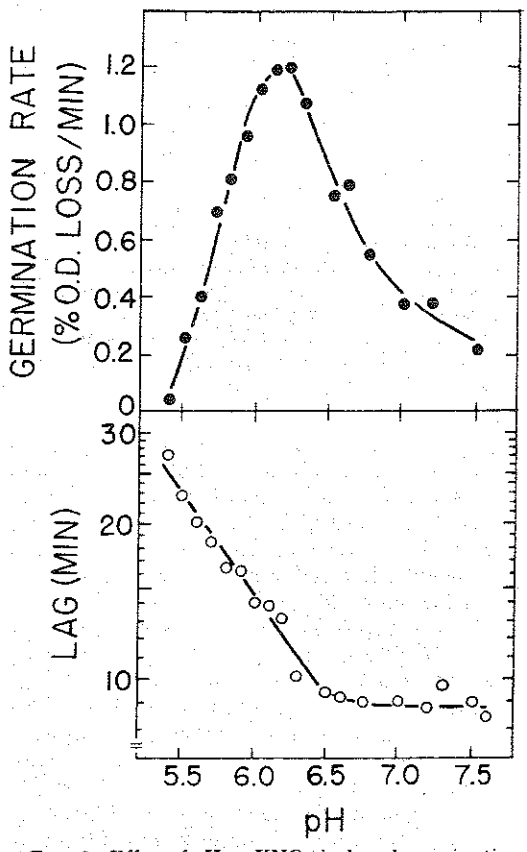

FIG. 2. *Effect of pH on KNO_3-induced germination of spores of* C. perfringens *NCTC 8238. Germination rate and lag of spores, incubated at various pH levels at 30°C in the standard germination mixture, were calculated from plots similar to that in Fig. 1.*

TABLE 1. *Germination of spores of* C. perfringens *NCTC 8238 in the standard KNO_3 germination mixture and the effect of this primary incubation for 1 h on subsequent secondary germination at 30°C*[a]

Temp (°C)	Incubation						
	Primary						Secondary
	OD loss (%)				GL	GR	GR
	1 h		2 h				
	Buffer control	KNO_3-induced	Buffer control	KNO_3-induced			
0	1.9	0.4	2.6	2.4	0.0	0.0	1.13
15	3.4	1.0	4.2	4.8	47.9	0.04	ND
17.5	4.1	2.8	5.6	9.6	37.6	0.14	ND
20	4.8	4.6	6.5	14.1	32.4	0.19	ND
22	4.5	9.3	6.4	22.5	24.8	0.29	0.91
23.5	4.6	22.0	6.2	35.6	22.8	0.63	0.91
25	5.2	26.8	6.7	37.1	19.4	0.76	ND
27.5	6.0	34.2	8.0	42.3	16.4	1.08	ND
30	6.2	40.5	8.4	45.5	12.8	1.25	—
32.5	5.0	41.9	6.5	46.6	9.8	1.41	ND
35	7.1	44.3	8.6	47.5	8.3	1.40	ND
37.5	5.9	32.0	7.2	36.0	7.4	0.98	ND
40	5.5	21.5	7.5	26.0	5.5	0.71	0.36
42.5	5.4	14.9	6.6	22.3	2.5	0.42	ND
45	5.7	7.3	8.1	13.0	1.8	0.18	0.43
47	6.1	4.7	8.5	5.6	1.3	0.08	ND
50	4.3	2.4	6.8	3.1	ND	0.03	0.48
60	4.3	1.9	6.6	3.1	ND	0.03	0.67
65	6.2	1.4	ND	ND	ND	ND	1.64
70	10.3	0.5	ND	ND	ND	ND	1.68

[a] Primary incubation was in standard sodium phosphate-KNO_3 germination mixture (legend to Fig. 1). Buffer control (sodium phosphate buffer, pH 6.1; no KNO_3) and KNO_3-induced (corrected for control) OD losses after 1 and 2 h of incubation at the primary incubation temperatures are shown. Incubation at the primary temperature for 1 h was followed, in some cases, by a secondary incubation at 30°C. Germination lag (GL), in minutes, calculated as in Fig. 1, was determined during primary incubation. Germination rate (GR), as percent OD lost per minute, was determined during both primary and secondary incubation periods. ND, no data obtained.

dark under phase optics. However, at this temperature, these germinated spores, being more heat sensitive than dormant spores, were inactivated or injured before a substantial decrease in OD had occurred, acquisition of heat sensitivity preceding loss of OD during germination (12). Indeed, even at 40°C, the rate of OD loss had declined abruptly before the 1-h primary incubation had been completed. Further, at 50°C, the ungerminated spores would not be additionally heat activated for germination during the secondary incubation (11). At 60°C, in contrast, fewer spores (14%) were germinated in buffered KNO_3, and although these were rapidly inactivated or injured, there *was* some additional heat activation of the ungerminated spores. After primary incubation at 60°C, there were more ungerminated spores poised to germinate than after primary incubation at 50°C. Similarly, after primary incubation for 1 h at 65 or 70°C, where only 6% germination had occurred, the germination rate during secondary incubation at 30°C was significantly higher than that of spores held at 60°C, or even at 0°C, during primary incubation (Table 1). Even less germination-inactivation-injury and even more heat activation had occurred at 65 or 70°C than at 60°C. We speculate that spores maintained at 60°C on a serving table in a medium suitable for germination (e.g., in foods) can be heat activated (11) and a certain percentage of them will germinate, but these germinated spores will be rapidly inactivated. When transferred to a lower, more suitable germination temperature, the remaining ungerminated spores will germinate rapidly and, under favorable conditions, may grow and be a health hazard.

Spore concentration. Germination rate was

optimal at about the standard spore concentration of 0.325 mg of spores per ml and decreased to ca. 70% of the standard rate when the spore concentration was decreased to 20% of the standard or increased to 5 times the standard. With increase in spore concentration from 20% to 40% of the standard, germination lag decreased from ca. 1.5 times that under standard conditions to a lag equal to that obtained under standard conditions and remained equal to the standard lag with further increase in spore concentration to 5 times the standard.

KNO_3 concentration. Germination rate was maximal (ca. 4 times that with the standard 5 mM KNO_3 concentration) at 100 mM KNO_3 (Fig. 3). With yet higher KNO_3 concentrations, the rate decreased, declining to approximately standard germination rate with 500 mM KNO_3. The effect of KNO_3 concentration on germination lag was less marked. Increasing the KNO_3 concentration from 0.5 mM to 50 mM was accompanied by a modest decrease in lag, whereas at concentrations exceeding 100 to 150 mM the lag increased slightly.

Sodium phosphate concentration. There was a broad peak of germination rate with buffer concentrations between 0.5 mM and the standard 10 mM (Fig. 4). With 100 mM sodium phosphate, the rate was only one-tenth of that under standard conditions. Lags with from 2 to 30 mM sodium phosphate buffer were approximately equal. The increased lag with lesser sodium phosphate concentrations may be attributable to an inability to control pH at very low buffer concentration, rather than to sodium phosphate concentration per se.

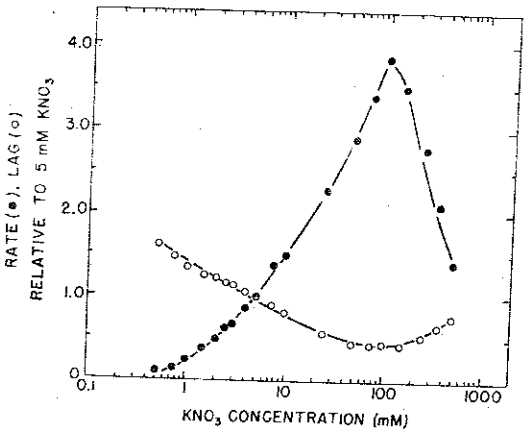

FIG. 3. *Effect of KNO_3 concentration on KNO_3-induced germination of spores of* C. perfringens *NCTC 8238. Germination rate and lag, calculated from plots similar to that in Fig. 1, are shown relative to these parameters under standard conditions (5 mM KNO_3, ◓).*

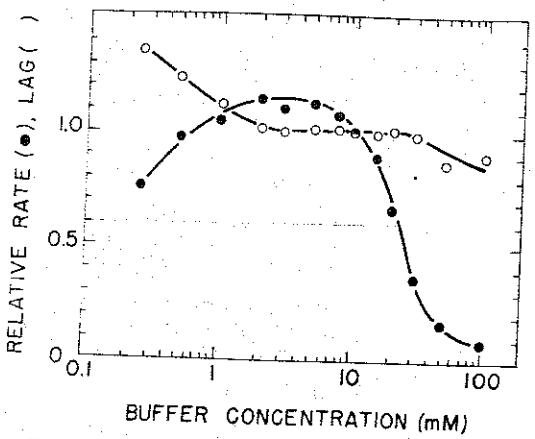

FIG. 4. *Effect of concentration of sodium phosphate buffer on the KNO_3-induced germination of spores of* C. perfringens *NCTC 8238. Germination rate and lag, calculated from plots similar to that in Fig. 1, are shown relative to these parameters under standard conditions (10 mM sodium phosphate, ◓).*

Spore investigators have frequently used lag as an index of the capacity of spores to germinate. It is of some interest to note that, in none of the conditions examined—pH, temperature, spore concentration, KNO_3 concentration, and sodium phosphate concentration—were the rate and lag responses equivalent as indices of germination. We believe that, if one must select a single criterion of germinability, germination rate would be the most useful and valid such criterion.

With our system, then, the conditions for maximum rate of KNO_3-induced germination were pH 6.1 to 6.3, 32.5 to 35°C, 0.325 mg of spores per ml, 100 mM KNO_3, and 2 to 5 mM sodium phosphate. We now find that spores of *C. perfringens* NCTC 8238 germinate equally well under air and under N_2. The ratio of germination rate under aerobic conditions to that under anaerobic conditions (flushed with N_2) was ca. 1.0. Similarly, the rate, lag, and extent of germination of spores of *C. sporogenes* (putrefactive anaerobe PA 3679h) were virtually equal under anaerobic or aerobic conditions (15). This, we believe, is analogous to the capability of the spores of the strict aerobe *B. megaterium* to germinate under anaerobic conditions (9). The duality of lack of necessity for aerobiosis in *B. megaterium* spore germination and lack of a requirement for anaerobiosis for *C. perfringens* spore germination suggests the generality of the thesis that germination and outgrowth are processes with separate and definable nutritional requirements and metabolic pathways. In addition to exploring this thesis, we plan to investigate more fully the effect of anions, other

than NO_3^-, and of cations, other than K^+, on *C. perfringens* spore germination.

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